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# **Long non-coding RNAs – Novel regulators of the Immune Response?**

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## **Abstract**

It is increasingly clear that much of the genome is transcribed to produce long non-coding RNAs (lncRNAs) and that these regulate biological responses by a diverse range of mechanisms. In the field of immunology, recent publications have shown widespread changes in the expression of lncRNAs during the activation of the innate immune response and T cell development, differentiation and activation. These lncRNAs control important aspects of the immune response such as production of inflammatory mediators, differentiation and cell migration through regulating protein-protein interactions or via their ability to base pair with RNA and DNA. In this review, we summarise what is presently known about the expression, function and mechanism of action of these immune-related lncRNAs.

## **What are long non-coding RNAs?**

The first draft of the human genome uncovered a number of surprises including the observation that exonic regions of protein coding genes represented < 2% of the genome. Whilst some of the remaining DNA plays a crucial role in the maintenance of DNA structure and regulation of mRNA expression (i.e. transcription binding sites, promoter and enhancer regions), subsequent studies have shown that a significant proportion is transcribed into 'non-coding RNAs'. Indeed, the recent release of the Encyclopedia of DNA Elements (the 'ENCODE' project aims to catalogue all the functional elements in human DNA) has concluded that ~ 80% of DNA is functional and importantly, that the majority (~62%) is transcribed into non-coding RNA (ncRNA) [1,2]. However, the broad definition employed to identify functional/transcribed regions of DNA which included an association with modified histones, methylated CpG dinucleotides or open chromatin, means this is likely to be an over-estimate [3,4]. Indeed, examination of evolutionary conservation gives a more conservative estimate of ~10% functional DNA although this still allows for large regions that might be transcribed into ncRNA [3,4].

ncRNAs are broadly classified as either short ncRNAs (< 200 nucleotides) or long ncRNAs (> 200 nucleotides). The microRNA (miRNA) family of short ncRNAs are best characterised and are known to induce mRNA degradation or block mRNA translation via the RNA interference pathways. In contrast much less is known about long non-coding RNAs (lncRNA) although comparison with mRNAs, has shown that these are generally shorter in length, contain fewer albeit longer exons and are expressed at lower levels (median is ~ 10% of mRNAs) [1,5,6]. lncRNAs also demonstrate low evolutionary sequence conservation, with estimates that only ~ 15% of mouse protein-coding genes have homologs in humans, leading to speculation that the majority of lncRNAs are non-functional [6-9]. The most recent release from GenCode (version 19) has annotated ~

14000 lncRNA genes in humans [5] although given that many lncRNAs are expressed in a cell-, tissue- and developmentally-stage specific manner, this is likely to be a significant underestimate [1,10]. Presently, lncRNAs are classified by their position relative to protein coding mRNAs and comprise the long intergenic ncRNA (lincRNAs), intronic lncRNA, antisense lncRNA, transcribed pseudogene lncRNAs and enhancer RNAs (eRNAs) (see Box 1). However, these arbitrary definitions will need refining in the light of the increasing volume of sequencing data and the accumulating information on lncRNA function and mechanism.

There is now accumulating evidence to indicate that lncRNAs are important regulators of physiological and pathological response [11,12]. However, their potential importance in the immune response is just emerging and it is this question that represents the topic of this review. As described in the forthcoming sections, immune-related lncRNAs are generally identified through examination of differential expression in response to activation of immune cells. This provides the basis for the subsequent functional and mechanistic analysis of individual lncRNAs. For clarity, these individual lncRNAs will be reviewed under the headings of innate immunity, acquired immunity, host-pathogen interaction and enhancer RNAs. However, before proceeding it is worth highlighting emerging trends surrounding the mechanism of action of these immune-related lncRNAs.

### **Mechanism of action of immune-related long non-coding RNAs**

An interesting observation from the sequencing data is that many of the immune-related lncRNAs are located close to, or partially overlapping, the 5' end (upstream) or 3' end (downstream) of protein coding genes implicated (and differentially expressed) in the immune response [13,14]. These are commonly transcribed in the antisense direction

(relative to the protein coding gene), which in the case of upstream lncRNAs, can produce bidirectional transcription of the lncRNA and mRNA from a shared promoter region. Confusingly, these are often termed antisense lncRNAs despite their being only partial or no overlap with protein-coding genes and as a result, we have recently proposed that these should instead be classified as mRNA-flanking lncRNAs (mf-lncRNAs) [13]. Given their location, this group which includes *THRIL* (TNF $\alpha$  and hnRNPL related immunoregulatory lincRNA) [15], *PACER* (p50-associated COX-2 extragenic RNA) [16], *lincR-Ccr2-5'AS* [14], *lnc-IL7R* [17] and *IL1 $\beta$ -RBT46* [13], have been shown to regulate the expression of their adjacent protein-coding gene *in cis*. eRNAs have also been demonstrated to control expression of protein coding genes *in cis* although chromatin looping is thought to be responsible for bringing the distal enhancer regions into close proximity with the promoter regions. The majority of the remaining immune-related lncRNAs, which are located in intergenic regions, regulate the immune response *in trans*. In addition to their *cis* actions, the 4 mRNA-flanking lncRNAs *THRIL* [15], *lincR-Ccr2-5'AS* [14], *lnc-IL7R* [17] and *IL1 $\beta$ -RBT46* [13] have also been shown regulate the expression of multiple additional genes *in trans*.

As with proteins, it is speculated that lncRNAs are composed of domains that permit either protein binding and/or base-pairing with RNA or DNA sequences [18-22]. Presently, it would appear that the action of most immune-related lncRNAs is mediated through protein binding. Targets include splicing factor proline/glutamine rich (SFPQ) [23], importin-beta family [24] and the transcription factors, nuclear factor- $\kappa$ B (NF- $\kappa$ B) [16,25], signal transducer and activator of transcription 3 (STAT3) [26] and the glucocorticoid receptor (GR) [27]. In these known examples, lncRNAs have been shown to act as 'decoys' to prevent protein-DNA (SFQR, NF- $\kappa$ B and GR) or as antagonists of protein-protein interactions (importin-beta and STAT3). Immune-related lncRNAs have also

known to interact with members of the heterogeneous nuclear ribonucleoproteins (hnRNPs) family [15,28] and components of the chromatin modifying complexes including the polycomb repressor complex 2 (PRC2) [29], WD repeat domain 5 (WDR5), a core subunit of the MLL methyltransferase complex [30] and the UTX/JMJD3 demethylases [31]. Although the exact role of these lncRNAs is undefined, it has been speculated that they might act as scaffolds to bring together proteins and/or target these to the DNA through base-pairing, a situation that has been described with other chromatin associated lncRNAs [12].

### **Long non-coding RNAs regulate the innate immune response**

The first evidence of a potential role for lncRNAs in the innate immune response was a report by Guttman *et al* [32] who used the intergenic deposition of epigenetic marks to identify 20 lincRNAs induced in LPS-stimulated mouse bone-marrow-derived dendritic cells (BMDD). Microarray and RNA sequencing (RNA-Seq) has now demonstrated differential lncRNA expression following activation of monocytes, macrophages, dendritic cells and fibroblasts, as well as viral infection in mouse lungs [15,25,26,28,33-35] (Table 1). As outlined in the following sections, investigators have then examined the role of individual lncRNAs in the innate immune response.

#### *LincRNA-Cox2 mediates the induction and repression of gene expression in mouse macrophages*

Initially identified by Guttman *et al* [32] and located 50kb downstream from the *Cox2* (*Ptgs2*) gene, *lincRNA-Cox2* was demonstrated to repress expression of 787 genes in non-stimulated BMDM and induce an increase in 713 genes following exposure to

Pam3CSK<sub>4</sub> (TLR1/2 agonist) [28] (Figure 1A). Gene Ontology (GO) enrichment analysis showed these groups were enriched for genes involved in the immune response and included *Ccl5* and *Il6*. Although the precise mechanism is unknown, the repressive action of *lincRNA-Cox2* was mediated through interaction with hnRNP-A/B and hnRNP-A2/B1 [28]. These hnRNPs are members of a family of multifunctional RNA binding proteins that are known to have a role in the processing of precursor RNA into mature mRNA, as well as regulating gene expression [36]. Of relevance, Sauvageau *et al* [37] have recently reported the production of a *lincRNA-Cox2* knockout mouse which will provide an invaluable resource for the *in vivo* studies. Limited preliminary analysis have shown that *lincRNA-Cox2* is selectively expressed in lung and found that knockout does not impact upon the development of these animals [37].

*THRIL regulates TNF $\alpha$  release and global gene expression in human monocytic THP-1 cells*

*THRIL* is located downstream of the gene encoding the BRI3 binding protein (*Bri3bp*), is transcribed from the opposite strand and partially overlaps the 3'-end of *Bri3bp*. Expression of *THRIL* in human monocytic THP-1 cells is reduced in response to Pam3CSK<sub>4</sub> although this effect was indirect and mediated through the autocrine/paracrine TNF $\alpha$  release. *THRIL* knockdown reduced expression of 444 genes (only 10 genes were increased) in non-stimulated cells and blocked differential expression of 317 of the 618 genes seen in response to Pam3CSK<sub>4</sub> including multiple inflammatory genes such as *IL6*, *CXCL8*, *CXCL10*, *CCL1* and *CSF1* [15]. As with *lincRNA-Cox2*, *THRIL* was shown to interact with hnRNPL, with the resultant complex binding to the TNF $\alpha$  promoter and driving transcription in both control and Pam3CSK<sub>4</sub>-stimulated THP1 macrophages (Figure 1B).



### *Lethe blocks NF- $\kappa$ B-driven inflammatory responses in mouse fibroblasts*

Rapicavoli *et al* [25] identified a large increase in expression of the *Rps15a-ps4* pseudogene following TNF $\alpha$ -induced activation of mouse embryonic fibroblasts (MEF). This pseudogene, renamed *Lethe*, was also induced following exposure to IL-1 $\beta$  and the anti-inflammatory glucocorticoid receptor (GR) agonist, dexamethasone [25]. *Lethe* was shown to block NF- $\kappa$ B (p65 or relA)-DNA binding and by this mechanism inhibit both the inflammatory response to TNF $\alpha$  and IL-1 $\beta$ , and promote the anti-inflammatory actions of dexamethasone [25] (Figure 1C).

### *NEAT1 mediated SFPQ relocation into nuclear paraspeckle promotes CXCL8 expression in response to viral infection*

The lncRNA *NEAT1* has a key role in regulating nuclear paraspeckle body formation [38,39]. Under basal conditions, paraspeckles contain a number of proteins including SFPQ, which are bound to *NEAT1*. However, in addition to *NEAT1*, SFPQ is also known to repress the transcription of CXCL8 through binding within its promoter region. Following reports that *NEAT1* expression is increased after viral infection of mouse neural cells [40], Imamura *et al* [23] examined the role of *NEAT1* in the antiviral response within HeLa and A549 epithelial cells. Activation of TLR3 using poly(IC) or following infection with influenza virus and herpes simplex virus was shown to stimulate *NEAT1* expression, resulting in *NEAT1*-dependent increases in paraspeckle formation [23]. Significantly, increased CXCL8 expression also occurred as a result of this translocation of SFPQ from the CXCL8 promoter sites and into the paraspeckles, (Figure 1D). Of relevance, knockdown and overexpression identified 85 additional genes that were regulated by *NEAT1*, including a number that are involved in the antiviral response [23].

### *Lnc-DC regulates differentiation of human monocytes into dendritic cells*

Profiling of lncRNA expression during differentiation of monocytes into dendritic cells identified *LOC645638*, renamed *lnc-DC*, as being uniquely up-regulated [26]. *lnc-DC* knockdown impacted upon the expression of 664 protein coding genes, resulting in impaired antigen uptake, reduced allogenic CD4<sup>+</sup> T-cell production and attenuated cytokine release. Interestingly, *lnc-DC* is located within the cytoplasm and its action is mediated through activation of STAT3, a transcription factor that is important to dendritic cell differentiation. Detailed analysis showed an interaction between the 3'-end of *lnc-DC* (265-417) and the C-terminus of STAT3 (residues 583 to 770), that prevented dephosphorylation of the Tyr705 (Y705) by Src homology region 2 domain-containing phosphatase-1 (SHP-1) and maintained STAT3 in its active phosphorylated form (Figure 1E).

#### *PACER mediates COX-2 expression in human monocytes*

*PACER* is located directly upstream of the *Cox2* transcriptional start site and expressed in the antisense direction [16]. Increased *PACER* expression, following PMA-induced differentiation of the monocytic U937 cell into macrophages and subsequent LPS-stimulation, was required for PMA/LPS-induced COX2 expression. This action was mediated through an interaction between the *PACER* and the inhibitory p50 homodimer (of NF- $\kappa$ B). *PACER* decreased p50-p50 occupancy at the *Cox2* promoter and permitted the binding of the active p50-p65 form of NF- $\kappa$ B and assembly of the RNA polymerase II pre-initiation complex [16] (Figure 1F). This event was associated with the recruitment of p300 histone acetyltransferase and increases in histone acetylation. Of relevance, these researchers demonstrate that baseline *PACER* expression and access to the *Cox2* promoter was established and maintained by the chromatin boundary/insulator factor CTCF, which opened the chromatin structure in this region [16].

In summary, these reports have described widespread changes in lncRNA expression following activation of the innate immune response and shown that these can regulate gene expression, the production of inflammatory mediators and the differentiation of monocytes into macrophage and dendritic cells. In addition to the innate immune response, publications have also begun to reveal a role for lncRNAs in T cell biology and the adaptive immune response.

### **Long non-coding RNAs regulate T cell activation, development and differentiation**

Although the existence of individual lncRNAs in T cells, including the noncoding transcript in CD4<sup>+</sup> T cells (*NTT*) [41], growth-arrest-specific transcript 5 (*Gas5*) [42,43] and *NRON* [24] (Table 1), has been known for a number of years, the first widespread screen was undertaken in human and mouse CD8<sup>+</sup> T cells by Pang *et al* [44]. Using microarrays, these investigators uncovered 100s of lymphoid-specific lncRNAs that showed altered expression during CD8<sup>+</sup> T cell activation and following differentiation into CD8<sup>+</sup> memory and effector T cells [44]. More recently, a comprehensive analysis of lincRNA expression during development and differentiation of 42 mouse T cell subsets identified 1524 lincRNA genes [14]. Focusing on the polarization of CD4<sup>+</sup> T cells into T<sub>H</sub>1 and T<sub>H</sub>2 subsets, they showed that expression of T<sub>H</sub>1 specific lincRNAs was preferentially induced by the T<sub>H</sub>1 related transcription factors, STAT4 and T-box transcription factor (T-bet). Similarly, the T<sub>H</sub>2 specific transcription factor STAT6 and GATA binding protein 3 (GATA3) preferentially regulated T<sub>H</sub>2 lincRNA expression. Once again, there is now merging evidence linking individual lncRNAs with T-cells function.

*NRON represses nuclear translocation of NFAT in resting T cells*

Nuclear factor of activated T cells (NFAT) is a  $\text{Ca}^{2+}$ -activated transcription factor that is an important mediator of T cell activation including the induction of IL-2 expression. The noncoding repressor of NFAT (*NRON*), an intronic lncRNA, was first identified in 2005 during an shRNA library screen against 512 lincRNAs that had been characterised in the mouse genome and shown to have significant homology to humans [24]. Mechanistic studies showed that interaction between *NRON* and karyopherin importin-beta-1 (KPNB1), blocked the nucleocytoplasmic transport and therefore the transcriptional activity of NFAT [24] (Figure 2A). Subsequent studies in resting cells indicated that the heavily phosphorylated NFAT is located within a large cytoplasmic RNA-protein complex that contains *NRON*, a scaffold protein - IQ motif containing GTPase activating protein (IQGAP) - and three NFAT kinases (casein kinase 1 (CK), glycogen synthase 3 (GSK) and dual specificity tyrosine phosphorylation regulated kinase (DYRK)) [45]. Knockdown studies showed a functional synergy between *NRON* and IQGAP1 in blocking NFAT dephosphorylation, a process that is required for nuclear translocation/activation and induction of IL-2 [45] (Figure 2A). *NRON* is therefore a constitutively expressed, intronic lncRNA that forms a complex with other proteins to bind the inactive NFAT and localise this transcription factor in the cytoplasm.

#### *NeST/Tmevpg1 induces IFN $\gamma$ expression in T cells*

Nettoie Salmonella pas Theilers's (*NeST*), which translates to 'clean-up Salmonella but not Theiler's', is a lncRNA that is located ~45kb downstream and transcribed in a convergent manner to the interferon(IFN)- $\gamma$  gene in mice [46]. *NeST*, which was formally known as *Tmevpg1*, is expressed in  $\text{T}_{\text{H}1}$   $\text{CD4}^{+}$  T cells,  $\text{CD8}^{+}$  T cells and natural killer cells [46]. Earlier work showed that expression of *NeST* was increased in  $\text{T}_{\text{H}1}$  but not  $\text{T}_{\text{H}2}$  polarised cells, correlated with *Ifng* expression and was dependent upon the  $\text{T}_{\text{H}1}$  specific

transcription factors, STAT4 and T-bet [47]. By using mouse strains over-expressing *NeST*, Gomez *et al* [30] showed increased clearance of *Salmonella* infection but reduced resistance to Theiler's virus, a mouse picornavirus. Mechanistic analysis indicated that nuclear located *NeST* induced the expression of *Ifng* *in trans* in activated CD8<sup>+</sup> T cells through an interaction with WDR5, a core subunit of the MLL H3K4 methyltransferase complex leading to histone methylation at the *Ifng* locus [30] (Figure 2B). Overall, this milestone publication is the first to have shown that lncRNAs play a central role in regulating the adaptive immune response using an *in vivo* infection model.

#### *lincR-Ccr2-5'AS regulates mouse T<sub>H</sub>2 migration into the lung*

The T<sub>H</sub>2 specific *lincR-Ccr2-5'AS*, which is located at the 5' end of *Ccr2* and transcribed in the antisense direction, was identified during lncRNA expression profiling of 42 T cell subsets in mice [14]. shRNA-mediated *lincR-Ccr2-5'AS* knockdown (delivered via lentivirus) in mouse T<sub>H</sub>2 cells resulted in the upregulation of 709 mRNAs that were shown to be preferentially expressed in T<sub>H</sub>2 cells and enriched for genes involved in the immune response. There was also down-regulation of 656 genes associated with the cell cycle and nuclear division [14]. Its mechanism of action is currently unknown although knockdown had no effect upon H3K4me3 levels, DNase hypersensitivity and RNA polymerase II binding thus indicating that *lincR-Ccr2-5'AS* does not function by modifying epigenetic marks and chromatin accessibility (Figure 2C). Given that *lincR-Ccr2-5'AS* expression was highly correlated with 7 of the 23 genes implicated in chemokine-mediated signaling pathways, including 6 that were located in the same genomic region, these investigators proceeded by examined its role in cell migration. Interestingly, they found that *lincR-Ccr2-5'AS* knockdown in T<sub>H</sub>2 cells impaired migration of T<sub>H</sub>2 cells to the lung.

Once again, early publications indicate that lncRNAs have an important role in mediating the differentiation, activation and migration of T-cells. Crucially, the report by Gomez *et al* [30] is the first demonstration that lncRNAs can regulate the immune response in an animal model of infection. In comparison, little is known about B-cell function although Bolland *et al* [48] has described a role for lncRNAs in the chromatin remodelling associated with the variable, diversity and joining (V(D)J) recombination required to produce antigen receptors (Ig or TCR). A subsequent publication has shown that transcription of these antisense and sense lncRNAs is linked to the looping of the V<sub>H</sub> regions into close proximity with the DJ<sub>H</sub> region during recombination in pro-B cells [49]. The process occurs within transcription factories although the mechanism has yet to be defined [49].

### **Long non-coding RNAs in host-pathogen interactions**

Many pathogens produce lncRNAs which are believed to be important both in the pathogen life-cycle and/or in the interaction between intracellular pathogens and their host cells. An example of the former is *NEAT1*, which controls HIV-1 replication by regulating the nuclear-to-cytoplasmic export of Rev-dependent instability element (INS) containing HIV mRNA [50]. Another well characterised example is polyadenylated nuclear (*PAN*) RNA produced by Kaposi's sarcoma-associated herpesvirus (KSHV) that is able to modulate viral gene expression as well as subvert the host immune response. Thus, induction of *PAN* RNA is important in the switch from latent to lytic infection [51,52], a process mediated by an interaction between *PAN* and the demethylases UTX and JMJD3 to remove the suppressive H3K23me3 mark within the KSHV viral genome (Figure 3A) [31]. Of note, *PAN* also physically interacts with LANA (latency associated nuclear

antigen), a protein that maintains latency by binding to the KSHV genome, and has a role in regulating the dissociation of the LANA protein upon viral activation [53]. In addition to regulating the viral life cycle, *PAN* RNA also suppressed the expression of host genes involved in the inflammatory and anti-viral response [52], a process that is mediated through activation of the polycomb repression complex 2 (PRC2) (Figure 3A) [29]. Overall, these studies imply that the viral non-coding *PAN* RNA is a regulator of both viral and host gene expression.

### **Enhancer RNAs are required for gene expression in mouse macrophages**

Enhancer regions regulate the expression of protein-coding genes, often over large distances and in an orientation-independent manner. These regions are characterised by increased sensitivity to nuclease, p300/CBP acetyltransferase and RNA polymerase II (RNAP) binding, as well as deposition of chromatin marks including histone H3K4 monomethylation (H3K4me1) and H3K27 acetylation (H3K27ac) [54]. Recent studies have also demonstrated that these sites are associated with active transcription to produce enhancer RNAs (eRNAs) that have a length ~ 0.5 - 5kb [1,22,55,56]. Of relevance, the recent release of the FANTOM 5 consortium has identified > 43000 eRNAs across 808 cells/tissues [57]. However, until recently it was unclear as to whether eRNAs represented transcriptional noise or were instrumental in mediating the action of the enhancer regions. To address this question, Lam *et al* [58] investigated the role of eRNA transcription during Rev-Erb- $\alpha$  (nuclear receptor subfamily 1, group D, member 1; Nr1d1) and Rev-Erb- $\beta$  (nuclear receptor subfamily 1, group D, member 2; Nr1d2)-mediated gene repression in mouse macrophages, a process occurring through the recruitment of the nuclear receptor co-repressor (NcoR)-HDAC3 to promoter and enhancer sites. Chromatin

immunoprecipitation of Rev-Erb- $\alpha$  and  $\beta$  and subsequent sequencing, showed that the majority (90%) of binding sites were located > 1kb from transcription start sites and within putative enhancer regions. Subsequent global run-on sequencing revealed that 76% of these enhancers were transcribed. To provide direct evidence that transcription of these eRNAs was linked to mRNA expression, these investigators focused on two eRNAs and their nearest neighbouring genes; *Cx3cr1* and *Mmp9*. Knockdown of these eRNAs reduced expression of *Cx3cr1* and *Mmp9* but not that of other local genes (Figure 3B). Furthermore, cloning of the enhancer region revealed that whilst the core regions containing the transcription factor binding sites were able to enhance gene expression *in cis*, the magnitude of *cis* regulation was notably increased when the eRNA region was also cloned. Similarly, we recently described widespread expression of eRNAs following exposure of human monocytes to LPS and that LPS-induced expression of *IL1 $\beta$*  is dependent on a downstream eRNA that also appeared to regulate *CXCL8* expression [13].

In addition to regulating gene expression, the transcription of eRNAs may also be important in the establishment of active enhancer regions. This was demonstrated in a series of elegant experiments by Kaikkonen *et al* [59] that examined the sequential changes that occurred during the re-modelling of the enhancer landscape following activation of mouse bone BMDM. Specifically, Kaikkonen *et al* [59] examined the changes associated with the selection and activation of ~ 3000 new enhancer regions following exposure to the TLR4 agonist, Kdo2-lipid A (KLA). This showed an initial interaction at the enhancer site between the TLR4-induced p65 component of NF- $\kappa$ B and the macrophage specific transcription factors, PU.1 and C/EBP. However, the subsequent establishment of the enhancer region, through the acetylation of histone H4K5/8, was coupled to eRNA transcription. Thus transcription of eRNAs appears to play



a role both in the establishment of enhancer regions [59] and in the enhancer-mediated gene expression *in cis* [58]. This conclusion is supported by studies in other cell types [60-62], as well as the FANTOM 5 data which showed that eRNA expression was predominantly bidirectional and strongly correlated with enhancer activity [57].

## Concluding Remarks

In concluding, we need to return to the question of whether lncRNA are novel regulators of the immune response? As a starting point, investigators often address this question by using RNA sequencing to detect the differential expression of known and novel lncRNAs. However, as with all profiling studies, one of the great challenges is the identification of functionally relevant lncRNAs from the large lists that are commonly produced. This problem is compounded by the fact that lncRNAs generally demonstrate poor evolutionary sequence conservation, thereby preventing the use of this traditional approach for the identification of functionality. Interestingly, although this indicates that most lncRNAs are likely to be non-functional, even those lncRNAs that are known to be conserved across multiple species (such as *Malat1*, *Cyrano* and *Xist*) are not reported using existing alignment programmes [6]. This has led to speculation that these algorithms, which are based upon whole-genome alignments, may be an inappropriate approach for detecting conservation in rapidly evolving lncRNAs. This underlines the need for better bioinformatics tools that can be employed to identify 'conserved' lncRNAs and their homologs [6]. Despite these limitations, cell-based studies have identified a number of lncRNAs that regulate the innate immune response. Importantly, these studies have also characterised their mechanism of action and as this number increases, it is

hoped that this information can be employed to refine the bioinformatics tools required for the identification of functional sequences and/or structural motifs.

Given the complexity of the immune response, arguably the strongest evidence to support a role of lncRNAs would be provided by animal models of infection and/or disease. Indeed, animal models provide the only available approach for investigating many aspects of the acquired immune response. This therefore underlines the importance of the report by Gomez *et al* [30] that demonstrated a role for *NeST* in a mouse model of viral and bacterial infection. However, the lack of conservation across species will once again represent a major hurdle to the standard approach of extrapolating from mouse to human.

Before finishing it also worth mentioning two recent potential areas of interest related to lncRNA. Firstly, given the importance of miRNAs in the immune response [64], it will be important to determine whether lncRNAs and miRNA interact to regulate mRNA expression. This possibility is supported by reports showing that lncRNAs can reduce miRNA levels by acting either as sponges [19] or through base-pairing with primary miRNAs to block their processing into mature miRNAs [65]. Secondly, it has also been suggested that lncRNAs might actually code for peptides and small proteins [66,67], an intriguing prospect given the importance of peptides in the immune response [68,69].

Overall, as with miRNAs [64], there is emerging evidence that lncRNAs are important regulators of the immune response. It is likely that there are many additional immune related lncRNAs to be discovered and these will act via multiple different mechanisms. Future studies will need to examine whether aberrant lncRNA expression is also linked to the development of autoimmune and allergic disease, as well as the inflammation associated with many chronic diseases. Indeed, several studies have reported differential

expression of lncRNAs in various inflammatory conditions [15,70-75] although further work is required to determine whether lncRNAs have an active role in the pathogenesis.

## **Abbreviations**

ASO – antisense oligonucleotides

BMDM - bone-marrow derived macrophages (BMDM)

ChIP-Seq - chromatin immunoprecipitation and sequencing

eRNA – enhancer RNAs

hnRNP - heterogeneous nuclear ribonucleoproteins

HOXAIRM1 - HOX antisense intergenic RNA myeloid 1

Gas5 - growth arrest specific transcript 5

GR – glucocorticoid receptor

IRF - interferon regulatory factor

lncRNA – long non-coding RNA

ncRNA – non-coding RNAs

NEAT1 - nuclear paraspeckle assembly transcript 1

NeST - Nettoie Salmonella pas Theilers's or clean-up Salmonella but not Theiler's

NRON - noncoding repressor of NFAT

PACER – p50-associated COX-2 extragenic RNA

PAN - Polyadenylated nuclear

Pam3CSK4 – Palmitoyl-3-cysteiny-l-seryl-(lysyl)<sub>4</sub>

RNA-Seq – RNA sequencing

SFPQ - splicing factor proline/glutamine-rich

SHP-1 - Src homology region 2 domain-containing phosphatase-1

THRIL - TNF $\alpha$  and hnRNPL related immunoregulatory lincRNA

**Figure 1 Long non-coding RNAs in the innate immune response**

**(A)** *LincRNA-COX-2* is located 3' of the *COX-2* gene and expressed in response to Pam<sub>3</sub>Csk<sub>4</sub> stimulation of mouse bone-marrow derived macrophages. It has widespread effects on inflammatory gene expression, repressing the transcription of anti-

inflammatory genes in non-stimulated cells whilst promoting the expression of pro-inflammatory genes following Pam3Csk4 exposure via an interaction with hnRNP-A2/B1 and hnRNP-A/B [28]. **(B)** Using human THP1 macrophages, *THRIL* was identified as an anti-sense lncRNA (overlapping *Bri3bp*) that promotes TNF $\alpha$  transcription by forming a complex with hnRNPL and binding to the promoter of TNF $\alpha$ . [15]. *THRIL* is expressed basally however this is decreased in a negative feedback loop following Pam3Csk4-induced TNF $\alpha$  release [15]. *THRIL* has also been shown to regulate basal and Pam3Csk4-stimulated gene expression. **(C)** *Lethe* expression is induced in mouse embryonic fibroblasts following treatment with TNF $\alpha$ , IL1 $\beta$  and dexamethasone and prevents NF- $\kappa$ B binding to NF- $\kappa$ B response elements [25]. **(D)** In basal cells, SFPQ1 is bound to the CXCL8 promoter to repress its transcription, as well as to *NEAT1* lncRNA within the paraspeckle bodies. *NEAT1* expression is up-regulated upon viral infection, leading to an increase in the size of *NEAT1*-containing paraspeckle bodies, the relocation of SFPQ1 from the CXCL8 promoter and de-repression of CXCL8 transcription [23]. **(E)** *Lnc-DC* expression is required for differentiation of human monocytes into dendritic cells. *Lnc-DC* promotes STAT3 phosphorylation through inhibiting the action of Src homology region 2 domain-containing phosphatase-1 (SHP-1). **(F)** *PACER* is located upstream of the *Cox2* transcriptional start site and expressed in the antisense direction. *PACER* induced COX2 expression by removing the repressive action of the p50 homodimer (of NF- $\kappa$ B) bound at the *Cox2* promoter.

## Figure 2 Long non-coding RNAs in the adaptive immune response

**(A)** The NFAT transcription factor is held inactive in the cytoplasm as part of a complex including the lncRNA *NRON*. Upon T cell activation, several of the proteins and *NRON*

disassociate from the complex and increased intracellular levels of  $\text{Ca}^{2+}$  activate calcineurin to facilitate the dephosphorylation of NFAT and thereby allowing it translocate to the nucleus [24,45]. **(B)** *NeST* is an eRNA located downstream of  $\text{IFN}\gamma$ , which promotes the transcription of  $\text{IFN}\gamma$  in  $\text{T}_\text{H}1$   $\text{CD4}^+$  T cells,  $\text{CD8}^+$  T cells and natural killer cells in mice. *NeST* binds to the methyltransferase WDR5 leading to methylation of the *IFN* $\gamma$  promoter [30,47]. **(C)** *LincR-Ccr2-5'AS* positively regulates the expression of genes involved in immunity and defence but negatively regulates genes involved in cell cycle and nuclear division. Specifically, *lincR-Ccr2-5'AS* regulates the transcription of several chemokine receptors genes (located in the same loci as the *lincRNA-Ccr2-5'AS* gene) in mouse  $\text{CD4}^+$   $\text{T}_\text{H}2$  cells that are required for cell migration [14].

### **Figure 3 Viral long non-coding RNAs and enhancer RNAs**

**(A)** The viral lncRNA PAN recruits histone-modifying complexes to the KSHV genome to promote the switch from latent to lytic infection. PAN also regulates host gene expression through PRC2 to repress the inflammatory response and promote cell growth and survival [53,77] **(B)** The *Mmp9*- and *Cx3cr1*-eRNAs promote the transcription of *Mmp9* and *Cx3cr1* in mouse bone-marrow derived macrophages *in cis*. The nuclear receptors Rev-Erb- $\alpha$  and - $\beta$  repress the expression *Mmp9* and *Cx3cr1* by binding to the enhancers and inhibiting eRNA transcription [58].

### **Box 1 – Classification of long non-coding RNAs (includes Box 1 Figure)**

With limited information on their function and mechanism of action, lncRNAs are currently classified by their relative position to protein coding genes. In general, most lncRNA are

transcribed by RNA polymerase II (RNAP II) and are therefore capped, polyadenylated and commonly spliced. Antisense (AS) lncRNAs are transcribed across the exons of protein-coding genes from the opposite strand [78] whilst intronic lncRNAs are transcribed from intronic regions in either the sense or antisense orientation. The largest group are the intergenic lncRNAs (lincRNAs), that are located between protein-coding genes [32,79]. Two additional groups that could arguably be classified as lincRNAs, are transcribed pseudogenes and enhancer RNAs (eRNAs). Transcribed pseudogenes arise when a gene loses the ability to produce a protein, either through mutation or inaccurate duplication [80]. eRNAs are produced by either mono- and bi-directional transcription at intergenic enhancer regions and can be differentiated from other lincRNAs by the presence of high H3K4me1 marks in their promoter regions [61,81,82]. This contrasts with the members of the antisense, intronic, intergenic and pseudogene lncRNA families that are associated with epigenetic marks characteristic of protein coding genes (i.e. high H3Kme3 in the promoter region).

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